รายงานวิจัยฉบับสมบูรณ์

ผลของฮอร์โมนเพศหญิงเอสโตรเจนต่อการแสดงออกของยีนสารกระตุ้นการเติบโตในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน

The effect of estrogen on the expression of growth factors in THP-1 monocytes

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สารบัญ

บทคัดย่อ

ที่มาและวัตถุประสงค์: ฮอร์โมนโปรเจสเตอโรนและเอสโตรเจนมีระดับสูงขึ้นในระหว่างที่สตรีตั้งครรภ์และมีบทบาทสำคัญต่อระดับการตอบสนองของระบบภูมิคุ้มกันของมารดา นอกจากนี้ความไม่สมดุลย์ของเมตาโบลิซึมของสารกระตุ้นการเติบโตเป็นลักษณะที่พบได้ในโรคเหงือกอักเสบเป็นก้อนเนื้อโตในช่องปากของสตรีมีครรภ์ ดังนั้นโครงการวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของฮอร์โมนโปรเจสเตอโรนและเอสโตรเจนต่อการแสดงออกของยีนในระดับเอมอาร์เอ็นเอของ สารกระตุ้นการเติบโต 3 ชนิดได้แก่ วาสคิวลาร์ เอนโดทีเลียล โกรท แฟคเตอร์-เอ (วีอีจีเอฟ-เอ), เบสิก ไฟโบรบลาสต์ โกรท แฟคเตอร์ (บีเอฟจีเอฟ) และ ทรานฟอร์มมิ่งโกรท แฟคเตอร์-บีต้า (ทีจีเอฟ-บีต้า) ในเซลล์โมโนไซต์ชนิด ทีเอชพี-วัน ที่ตอบสนองต่อการกระตุ้นด้วยไลโปโพลีแซคคาร์ไรด์ (แอลพีเอส) จากเชื้อพอร์ไฟโรโมแนสจินจิวัลลิส

วัสดุและระเบียบวิธีการวิจัย: เซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน ที่เพาะเลี้ยงไว้จะถูกกระตุ้นฮอร์โมนโปรเจสเตอโรนและเอสโตรเจนหรือ แอลพีเอสจากเชื้อ อี โคไล และพอร์ไฟโรโมแนส จินจิวัลลิสเป็นเวลา 1, 3 และ 24 ชั่วโมง ทำการศึกษาระดับการแสดงออกของยีนในระดับเอมอาร์เอ็นเอของ สารกระตุ้นการเติบโต วีอีจีเอฟ-เอ, บีเอฟจีเอฟ และ ทีจีเอฟ-บีต้า ด้วยวิธี อาร์ที-พีซีอาร์ และวัดปริมาณการแสดงออกของยีน วีอีจีเอฟ-เอ และ บีเอฟจีเอฟ ด้วยวิธีเรียลไทม์ อาร์ที-พีซีอาร์ ตรวจสอบการเจริญเติบโตและความมีชีวิตของเซลล์ด้วยชุดตรวจการแบ่งตัวของเซลล์

ผลการวิจัย: ในรายงานวิจัยนี้พบว่าฮอร์โมนโปรเจสเตอโรนจะเพิ่มระดับการแสดงออกของยีน วีอีจีเอฟ-เอ ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน แต่ฮอร์โมนเอสโตรเจนไม่มีผลดังกกล่าว นอกจากนี้โปรเจสเตอโรนยังเสริมฤทธิ์ของแอลพีเอสจากเชื้อ พอร์ไฟโรโมแนส จินจิวัลลิส ในการกระตุ้นระดับการแสดงออกของยีน วีอีจีเอฟ-เอ ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับการกระตุ้นด้วยแอลพีเอส จากเชื้อ พอร์ไฟโรโมแนส จินจิวัลลิสเพียงอย่างเดียวอีกด้วย อย่างไรก็ดีทั้ง โปรเจสเตอโรนและเอสโตรเจน กลับไม่มีผลต่อการแสดงออกของยีนในระดับเอมอาร์เอ็นเอของ สารกระตุ้นการเติบโตชนิด เบสิก ไฟโบรบลาสต์ โกรท แฟคเตอร์ และ ทรานฟอร์มมิ่งโกรท แฟคเตอร์-บีต้า ในเซลล์โมโนไซต์ชนิด ทีเอชพี-วัน

สรุปผลการวิจัย: ผลเสริมฤทธิ์ของโปรเจสเตอโรนที่มีต่อการแสดงออกของยีนในระดับเอมอาร์เอ็นเอของ วีอีจีเอฟ-เอ อาจจะมีบทบาทในพยาธิกำเนิดของโรคเหงือกอักเสบเป็นก้อนเนื้อโตในช่องปากของสตรีมีครรภ์

คำสำคัญ: โปรเจสเตอโรน เอสโตรเจน วีอีจีเอฟ-เอ พอร์ไฟโรโมแนส จินจิวัลลิส โมโนไซต์

Abstract

Background/purpose: Progesterone and estrogen are elevated during pregnancy and play a role in maternal immune responses. In addition, unbalanced metabolism of growth factors has been demonstrated in pregnancy tumor. Therefore, we aimed to investigate the effect of progesterone and β-estradiol on vascular endothelial growth factor-A (VEGF-A) , basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) mRNA expression in THP-1 monocytes in response to *Porphyromonas gingivalis* lipopolysaccharide (LPS).

Materials and methods: THP-1 monocytes were incubated with progesterone, β-estradiol or LPS from *Escherichia coli* and *P.gingivalis* for 1, 3 and 24 h. The expression for VEGF-A, bFGF and TGF-β was investigated using conventional RT-PCR. VEGF-A and bFGF expression were measured using real-time RT-PCR. Cell growth was assessed using cell proliferation assay.

Results: We reported herein that progesterone, but not β-estradiol, increased VEGF-A mRNA expression in THP-1 monocytes. Significantly, progesterone enhanced VEGF-A mRNA expression in *P. gingivalis* LPS treated monocytes in comparison with a treatment with *P. gingivalis* LPS alone. However, neither β-estradiol nor progesterone had any effect on bFGF production at mRNA levels.

Conclusion: The enhancing effect of progesterone on VEGF-A mRNA expression may have a role in the pathogenesis of pyogenic granuloma in pregnant women.

Keywords: Progesterone; estrogen; VEGF-A; *P.gingivalis*; monocytes

Executive Summary

1. ความสำคัญและที่มาของปัญหา

โรคเหงือกอักเสบในสตรีมีครรภ์เป็นโรคปริทันต์ที่พบได้เป็นประจำ โดยพบว่าสตรีมีครรภ์กว่าร้อยละ 30 จะตรวจพบโรคนี้ในช่องปากได้ [1, 2]. ราบงานวิจัยหลายการศึกษากล่าวว่าโรคปริทันต์อักเสบในสตรีมีครรภ์ อาจมีความสัมพันธ์กับการเกิดอาการแทรกซ้อนต่าง ๆ ในการคลอดบุตรและอาจมีผลต่อสุขภาพของทารกได้ [3-7]. ในทางตรงกันข้ามก็พบว่าขณะสตรีตั้งครรภ์จะมีการเปลี่ยนแปลงของสุขภาพในช่องปาก และมีผลให้เพิ่มความเสี่ยงต่อการเกิดโรคเหงือกอักเสบได้เช่นกัน [6, 8]. เนื่องจากโรคปริทันต์อักเสบ และภาวะแทรกซ้อนต่าง ๆ ในการคลอดบุตรล้วนเป็นปัญหาสำคัญของระบบสาธารณสุขไทย การศึกษาวิจัยที่เกี่ยวข้องกับชีววิทยาภูมิคุ้มกันของทั้งสองภาวะนี้น่าจะเกิดประโยชน์การตรวจวินิฉัยและวางแผนป้องกัน รักษาความผิดปกติทั้งสองชนิดได้

อย่างไรก็ดียังไม่มีข้อมูลอธิบายว่าเหตุใดสตรีมีครรภ์บางรายจึงแสดงอาการของเหงือกอักเสบร่วมกับลักษณะเหงือกที่มีขนาดโตผิดปกติ รายงานวิจัยเสนอว่าฮอร์โมนเพศหญิงเอสโตรเจนน่าจะมีบทบาทสำคัญในการเกิดความผิดปกตินี้ เนื่องจากตรวจพบระดับของฮอร์โมนชนิดนี้ในน้ำลายที่ระดับสูงขึ้นอย่างมีนัยสำคัญระหว่างตั้งครรภ์ [4]. สารกระตุ้นการเติบโต) Growth factorsรายของแผลในอวัยวะปริทันต์โดยการมีหน้าที่สำคัญในการระบวนการห (ส่งเสริมการเจริญของเนื้อเยื่อบุผิว การสร้างใหม่ของเส้นเลือด และการสะสมของเส้นใยคอลลาเจน [9, 10]. โดยพบว่าร่างกายจะสร้างสารกระตุ้นการเติบโตชนิดต่าง ๆ ได้แก่ vascular endothelial growth factor-A (VEGF-A) , basic fibroblast growth factor (bFGF) และ transforming growth factor-β (TGF-β) อย่างไรก็ดีในสภาวะที่ร่างกายถูกกระตุ้นมากเกินไป การไม่สมดุลย์ของปริมาณสารกระตุ้นการเติบโต หรือมีการผลิตที่มากเกินความจำเป็นอาจเป็นสาเหตุหนึ่งของพยาธิกำเนิดของลักษณะเหงือกโตผิดปกติในสตรีมีครรภ์ เนื่องจากยังไม่มีรายงานการศึกษาถึงผลร่วมระหว่างฮอร์โมนเอสโตรเจนและแบคทีเรีย *P. gingivalis* ซึ่งเป็นสาเหตุก่อโรคปริทันต์อักเสบต่อการผลิตสารกระตุ้นการเติบโตในเนื้อเยื่อเหงือก ข้อมูลจากการศึกษาในเรื่องนี้จะเป็นประโยชน์อย่างยิ่งเพื่อเป็นข้อมูลในการอธิบายกลไกชีวโมเลกุลที่ฮอร์โมนเอสโตรเจนมีผลที่ไม่พึ่งประสงค์ต่อการตอบสนองของเซลล์ในร่างกาย

2. วัตถุประสงค์

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของฮอร์โมนเอสโตรเจนต่อการแสดงออกของยีนสารกระตุ้นการเติบโตในเซลล์ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน ที่ถูกกระตุ้นด้วย LPS จากแบคทีเรียชนิด *P.gingivalis* ในห้องปฏิบัติการ

3. ระเบียบวิธีวิจัย

3.1 การเพาะเลี้ยงเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน ในห้องปฏิบัติการประกอบด้วย

3.3.1 การเพาะเลี้ยงเพิ่มจำนวนและการเก็บเซลล์โดยการแช่แข็ง

3.3.2 การกระตุ้นเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน ด้วยวิตามิน D3

3.2 การวิเคราะห์การแสดงออกของยีนสารกระตุ้นการเติบโตในเซลล์ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน

3.2.1 การกระตุ้นเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน ด้วย LPS จากแบคทีเรียชนิด *P.gingivalis* และฮอร์โมนโปรเจสเตอโรนและเอสโตรเจน

3.2.2 การแยกปริมาณสารพันธุกรรมด้วยวิธี mRNA extraction และการสร้าง cDNA ด้วยวิธี reverse transcription

3.2.3 การทดสอบการแสดงออกของยีนด้วยวิธี polymerase chain reaction (RT-PCR)

4 ผลวิจัย

4.1 ฮอร์โมนโปรเจสเตอโรนจะเพิ่มระดับการแสดงออกของยีน วีอีจีเอฟ-เอ แต่ฮอร์โมนเอสโตรเจนไม่มีผลต่อระดับการแสดงออกของยีน วีอีจีเอฟ-เอ ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพีวัน-

4.2 โปรเจสเตอโรนเสริมฤทธิ์ของ แอลพีเอส จากเชื้อ พอร์ไฟโรโมแนส จินจิวัลลิส ในการกระตุ้นระดับการแสดงออกของยีน วีอีจีเอฟ-เอ ในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพีวันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับการกระตุ้นด้วยแอลพีเอส จากเชื้อ พอร์ไฟโรโมแนส จินจิวัลลิส เพียงอย่างเดียว

4.3 ทั้ง โปรเจสเตอโรนและเอสโตรเจน ไม่มีผลต่อการแสดงออกของยีนในระดับเอมอาร์เอ็นเอของ สารกระตุ้นการเติบโตชนิด เบสิก ไฟโบรบลาสต์ โกรท แฟคเตอร์ และ ทรานฟอร์มมิ่งโกรท แฟคเตอร์-บีต้า ในเซลล์โมโนไซต์ชนิด ทีเอชพี-วัน

5 ผลงานหัวข้อเรื่องที่คาดว่าจะตีพิมพ์ในวารสารวิชาการระดับนานาชาติ/

ชื่อเรื่องที่ตีพิมพ์ : Progesterone, but not β-estradiol, enhances *P.gingivalis* LPS induced-VEGF-A expression in human THP-1 monocytes.

ชื่อวารสารที่ตีพิมพ์ : Journal of Dental Sciences

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1 Introduction

Pregnancy gingivitis is a common form of periodontal disease that affects more than 30% of pregnant women [1, 2]. A number of studies suggest a bi-directional relationship between maternal periodontal diseases and adverse pregnancy outcomes[3-7]. On the one hand, the risk for periodontal diseases is increased when pregnancy is present[6, 8]. On the other hand, epidemiological studies have demonstrated that pregnant women with periodontal diseases or poor dental health had increased risk of having preterm birth (PTB) or low birthweight (LBW) baby [5, 11, 12]. PTB has dramatic impact on the health care system not only due to high mortality and morbidity but also due to significant medical costs. The prevalence rate of preterm birth is significantly increasing annually in many countries [5], including in Thailand [13]. As periodontal disease and PTB are considered as a global public health problem the knowledge regarding the immunobiology between these conditions could be a key to establish rational diagnostic and therapeutic strategies for periodontal disease associated with pregnancy. Although the clinical and histological characteristics of exacerbated gingival inflammation in pregnant individuals are well documented, its aetiology has not yet been clearly established and it is unknown why only some pregnant women develop frank signs of gingival inflammation with gingival enlargement. The function of female sex hormones could potentially link the causal relationship between pregnancy and pregnancy induced-gingival diseases. Indeed, the finding that salivary levels of estrogens and progesterone are elevated during pregnancy suggests a role for these hormones in the pathogenesis of pregnancy-induced gingival pathology[4].

Growth factors have a controlling role in periodontal wound healing and periodontal tissue remodeling by promoting epithelialization, angiogenesis and collagen synthesis [9, 10]. In wound healing process, host cells produce a myriad of growth factors such as VEGF-A, bFGF and transforming growth factor-β (TGF-β). However, the unbalanced/excessive production of growth factors may be one mechanism that mediates

the development of pregnancy-induced gingival enlagrement [14-16]. Several studies demonstrate an increase in levels of specific cytokines and growth factors in gingival overgrowth tissues[4, 17]. *In vitro*, it has been shown that estrogen is a potential regulator of the production of several growth factors such as TGF-β, FGF and nerve growth factor in different cell types[18-21]. It is thus plausible that estrogen may modulate the response to periodontal infection in term of growth factor synthesis. Since *Porphyromonas gingivalis* is a major bacterial species implicated in many form of gingival diseases, including pregnancy gingivitis[1, 22-24], the interaction between female sex hormones and *P. gingivalis* may be fundamentally important in the development of pregnancy related-gingival enlargement. In addition, there is no study that demonstrates the interacting role between sex hormones and oral bacteria such as *P. gingivalis* in the synthesis of growth factors in the gingival tissues. The data from this project will be useful to provide an insight into a possible cellular mechanism by which sex hormones might adversely modulate tissue responses. The present study will also expand our knowledge of the molecular interaction between sex hormones and the regulation of growth factors in human monocyte-stimulated with *P.gingivalis* LPS.

Aims

The aims of present study were to investigate the *in vitro* effect of female sex hormones, estrogen and progesterone on the expression of growth factors, i.e. VEGF-A, bFGF and TGF-β, in the human monocytic cell line THP-1-stimulated with *P.gingivalis* (Pg.) LPS.

2 Hypotheses

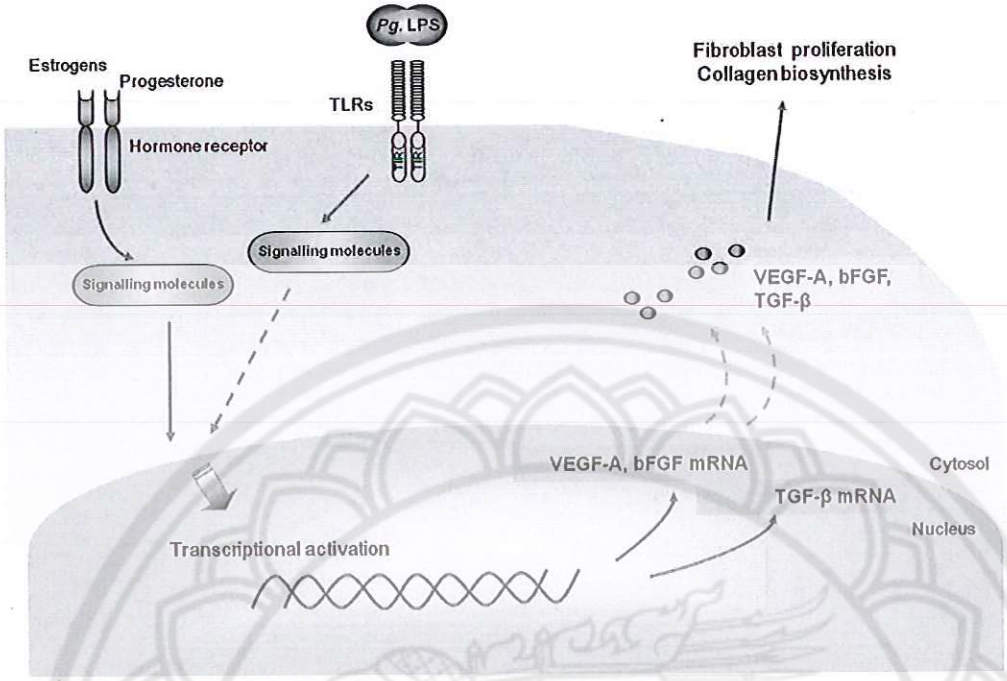


Figure 1. The schematic diagram of proposed effect of female sex hormones on LPS-induced the expression of growth factors.

The activation of Toll-like receptors (TLRs) by LPS generates signaling cascades converging on similar effects on host cells, resulting in expression of genes related to cytokine and growth factor synthesis. Female sex hormones activate the related signaling pathways, which in turn, also may result in enhancing in production of growth factors such as VEGF-A, bFGF and TGF-β. Subsequently, these growth factors may mediate the fibroblast proliferation and collagen biosynthesis which could contribute to the overgrowth of gingival tissues.

3 Literature review

Periodontal disease is a group of chronic inflammatory diseases that affect the supporting tissues of the dentitions. Gingivitis and periodontitis are the two common forms of periodontal diseases. Gingivitis, a more stable form, is an inflammatory condition of the soft tissue surrounding the teeth (the gingiva) without the involvement of the attachment apparatus whereas periodontitis involves the deeper periodontium resulting in the clinical attachment loss with the destruction of gingiva, periodontal ligament, cementum and alveolar bone (Kinane, 2001). Periodontal diseases are a global public health problem (Albandar and Rams, 2002), and in particular periodontitis, which is a major cause of adult tooth loss (Papapanou, 1996).

Another important form of periodontal diseases is pregnancy gingivitis. Pregnancy gingivitis usually develops between the second and eighth month of pregnancy and affects 36–100% of pregnant women [3, 7, 8]. Many cross-sectional and longitudinal clinical studies have demonstrated that the prevalence and severity of gingival inflammation increase during pregnancy [6, 25, 26]. In an experimental gingivitis study, a more severe development of gingival inflammation was observed during pregnancy than after delivery [27].

The clinical signs of pregnancy gingivitis include red swollen gingiva, increased gingival probing depths, increased bleeding upon probing or mechanical stimulation, increased gingival crevicular fluid flow and increased tooth mobility. The most affected area appears to be in interproximal sites of anterior teeth. Preexisting gingivitis or periodontitis has been noted to worsen dramatically in pregnant women [2]. However, there is no evidence confirmed that steroid sex hormone-induced gingivitis could proceed to periodontitis [8]. In addition to the gingival changes seen due to an enhanced inflammatory response during pregnancy, 0.5–9.6% of women who are pregnant also experience localized gingival enlargement consistent with pyogenic granulomas [28]. The pregnancy-associated pyogenic granuloma, or "pregnancy tumor" is a painless, exophytic mass that has either a sessile or pedunculated base extending from the gingival margin or from the interproximal tissues of the teeth. These conditions could adversely affect the functions of the dentitions.

It is now accepted that the development of periodontal disease is associated with a specific group of predominantly Gram-negative, anaerobic or microaerophilic bacteria. They colonize on the root surface in the subgingival area [29]. The key periodontal pathogens in periodontal diseases are *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola Aggregatibacter* (formerly termed *Actinobacillus*) *actinomycetemcomitans* and *Prevotella intermedia* [24, 30]. Although bacteria are essential, the bacteria alone are not sufficient for the disease to occur. Periodontal disease results from the complex interaction between host response and periodontal pathogens [31]. After colonization on the gingival sulcus by periodontal bacteria, the bacteria release their products, for example, lipopolysaccharide (LPS) which is a well known virulence factor of gram-negative bacteria. LPS has the potential to interact with nearly all cell types in periodontal tissues including immune cells and resident cells and thereby initiating a defence mechanism. The initial immune response in periodontal disease is characterized by the action of the innate immune system which, in this context, consists of the gingival epithelium, fibroblasts, neutrophils, dendritic cells, and monocytes/macrophages [32]. In fact, innate host recognition of LPS is a key initiating event for the subsequent clearance of gram-negative bacteria from infected host tissues [24].

One important component of innate immunity that plays a vital role in periodontal disease is monocytes. In response to inflammatory signals, monocytes can migrate quickly to sites of infection in the tissues and differentiate into macrophages which can effectively capture invading pathogens. The phagocytosis of bacteria by macrophages results in cytokine secretion and antigen-presentation to induce a more effective adaptive immunity [16, 32].

There is increasing evidence that suggests a bi-directional relationship between maternal periodontal diseases and adverse pregnancy outcomes [1, 5, 8, 12, 33]. The presence of pregnancy gingivitis in certain pregnant individuals may be recognized as indicator or predictive factor for higher risk of having preterm birth and may provide an indication for thorough health care and prevention in such conditions [33-35]. Therefore, a complete understanding how sex hormones contribute periodontal disease progression

could be a key to establish rational diagnostic and therapeutic strategies for periodontal disease associated with pregnancy.

Exploring the underlying mechanism how pregnancy contributes to periodontal disease highlights the link between these two conditions. Several biological mechanisms have been proposed to explain the pathobiology of the interaction between pregnancy and periodontal disease [8], including depression of the immune system, increased vascularity and vascular flow, cellular changes and changes in oral biofilms [1, 4, 27, 36, 37]. However, the precise mechanistic links between pregnancy and gingival changes have yet to be fully understood.

Alterations in immune-inflammatory process by hormonal imbalance have been proposed to explain the pathophysiolocal connections between periodontal disease and pregnancy. In addition, female sex hormones have been shown to play a vital role on the function of immune cells including monocytes/macrophages [18, 38, 39]. The sex hormone receptor is expressed in a number of immune cells such as neutrophils, mast cells, T and B lymphocytes and monocytes [18]. It has been demonstrated that female sex hormones have a pro-inflammatory effect on gingiva in response to periodontal pathogens. Estrogen and progesterone can activate the secretion of a myriad of cytokines and inflammatory mediators by immune and non-immune cells in the gingiva [18, 19]. In addition to the immuno-stimulatory effect of estrogen on a number of cell types in the gingiva, estrogen can also influence on cellular proliferation, differentiation and growth in target tissues, including keratinocytes and fibroblasts in the gingiva [8, 17, 37]. For example, estradiol can induce proliferation of gingival fibroblasts derived from pre-menopausal women [40]. In addition, both estradiol and progesterone were shown to be selectively accumulated by *P. intermedia* as a substitute for vitamin K and thus postulated to be acting as a growth

factor for this micro-organism [2]. These data likely suggest the possible role of estrogen in the development of gingival enlargement associated with pathogen-induced gingival inflammation.

Growth factors play an important role in wound healing and tissue repair during periodontal inflammation. In fact, growth factors potentiate the healing process by enhancing epithelialization, angiogenesis and extracellular matrix formation. In addition, it is evident that cytokines and growth factors levels are elevated in human drug-induced gingival overgrowth including interleukin-6 (IL-6), IL-1β, platelet-derived growth factor-B (PDGF-B), fibroblast growth factor-2 (FGF-2), transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF). In the inflammatory phase of wound healing, monocytes migrate to the wound site and differentiate into macrophages, and produce a variety of growth factors, which stimulate proliferation of fibroblasts, collagen deposition and re-vascularization [9]. However, overproduction of growth factors may result in the excessive fibroblast proliferation and collagen biosynthesis which could be one factor that contributes to the development of the clinical signs of pregnancy-induced gingival overgrowth. (Williamson *et al.*, 1994; Uzel *et al.*, 2001). One possible mechanism may stem from the immunomodulatory effects of the increased levels of female sex hormones during pregnancy. Indeed, hormonal imbalance is thought to be a major determinant that is responsible for the development of pregnancy gingivitis and pregnancy gingival overgrowth. The levels of serum sex hormones such as progesterone and estrogen are elevated throughout the 3 trimesters of pregnancy, reaching the highest levels during the third trimester [41]. Interestingly, the levels of both hormones in saliva also significantly increased during pregnancy [4]. Studies have shown that estrogen is a potential regulator of growth factor production. Estrogen at pregnancy levels were demonstrated to regulates the production of several growth factors in different cell types. For example, estrogen was found to enhance the induction of the TGF-β3 promoter in osteosarcoma cells [42] and in human kidney carcinoma cells [43]. In addition, estrogen stimulates FGF mRNA expression and its

secretion in fibroblasts derived from uterine endometrium [44]. Only a few studies have reported the effect of estrogen on the production of growth factors in monocytes/macrophages. In human macrophages, estrogen enhanced nerve growth factor production by up-regulation of AP-1 transcriptional activity [20]. In a recent study, estrogen was found to increase the protein expression of IL-6, TNF-α and TGF-β by human macrophages derived from activated monocytes when compared to untreated cells [21]. However, the information regarding the role of sex hormones in the regulation of growth factors in pregnancy-induced gingival inflammation is still not completely understood [17]. In addition, there is no study that investigates the interaction between sex hormones and the periodontal pathogens such as *P. gingivalis* in term of mRNA expression of growth factors in human monocytes. Such study will be useful to provide the underlying molecular mechanisms of the pathogenesis of pregnancy-induced gingival overgrowth. Therefore, the aim of present study is to investigate the *in vitro* effect of female sex hormones, estrogen and progesterone on the expression of growth factors (VEGF-A, bFGF and TGF-β) in the human monocytic cell line THP-1-stimulated with *P.gingivalis* LPS.

4 Materials and Methods

4.1 Materials

Progesterone (P7556), estrogen (β-Estradiol, E4389) and cell culture media were purchased from Sigma-Aldrich (St. Louis, USA). All plasticware were purchased from (Nunc A/S, Denmark). Vitamin D_3 (1α, 25-dihydroxy-vitamin D_3) was purchased from Calbiochem (Merck Chemicals, USA). Ultrapure LPS from *E. coli* 0111.B4 and ultrapure LPS from *P. gingivalis* were purchased from Invivogen (San Diego, USA). Recombinant prostaglandin E_2 (PGE_2) was purchased from R&D Systems (Minneapolis, USA).

4.2 THP-1 monocytes cell culture and stimulation

THP-1 pro-monocytes were purchased from the Cell Line Service (CLS, Germany) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2. Cells were maintained at a density of 3-8 x 10^5 cells/ml and cell viability was monitored using trypan blue exclusion, which was always >95%. THP-1 cells have a pro-monocyte phenotype with low levels of CD14 expression. Therefore prior to use in stimulation experiments we differentiated THP-1 promonocytes to a monocyte phenotype by incubation with 0.1 µM vitamin D_3 (VitD3) for 48 h, as previously reported[45]. It has shown that 100 ng/ml *E.coli/P.gingivalis* LPS is an optimal concentration used in stimulation experiments[45] and progesterone, β-estradiol and PGE_2 concentrations used in experiments are chosen based on previous studies applying a comparable experimental setup[46]. Cells were then stimulated with LPS from *E. coli* and *P. gingivalis* (100 ng/ml), progesterone (10 ng/ml), β-estradiol (1 ng/ml) or PGE_2 (2.5 ng/ml) for 1, 3 and 24 h.

4.3 RNA extraction and cDNA synthesis by Reverse Transcription

Total RNA was isolated from culture cells using a commercial RNA extraction kit (RNeasy® Mini Kit, Qiagen, Germany) according to the manufacturer's instruction. The total RNA concentration of each sample was measured with a spectrophotometer (Nanodrop, Thermo Fisher, USA) and then stored at -80 °C. An aliquot containing 1.0 µg of total RNA was used for the reverse transcription reaction, which was conducted using iScript™ cDNA Synthesis kit (Bio-Rad, USA). cDNA samples were then stored at 2-8 °C.

4.4 Conventional RT-PCR

The mRNA expression of VEGF-A and bFGF genes was detected using a conventional PCR method. β2 microglobulin (β2M) was used as an internal control gene. All primers used for PCR analysis were purchased from Intron Bio (Korea). The primer sets specific for each gene and the PCR conditions are summarized in Table 1. The PCR was performed using i-Taq™ PCR Master mix (Intron Bio, Korea) with 2.0 µl of cDNA sample. PCR amplification was conducted for 35 cycles and the products of the PCR were then separated by 2% agarose gel electrophoresis, visualized with ethidium bromide staining, and viewed under UV light.

Table 1 Primers used in the polymerase chain reaction expression analyses

Gene	Primer sequence (5'-3')	Annealing (^{o}C)	Temp.	Product size (bp)
VEGF-A [47]	F –ATGAACTTTCTGCTGTCTTGGGT	60		344
	R –TGGCCTTGGTGAGGTTTGATCC			
bFGF [48]	F –AGAGCGACCCTCACATCAAG	60		234
	R –ACTGCCCAGTTCGTTTCAGT			
TGF-β	F –GGGACTATCCACCTGCAAGA	60		239
	R –CCTCCTTGGCGTAGTAGTCG			
β2M	F –ACCCCCACTGAAAAAGATGA	60		120
	R –CTTATGCACGCTTAACTATC			

4.5 *Quantitative analyses for VEGF-A mRNA using real-time RT-PCR*

The quantification of VEGF-A mRNA levels was performed using a LightCycler® 480II detection system (Roche). Reaction mixtures for PCR (20 µl) were prepared by mixing 1.0 µl of cDNA solution, LightCycler 480 SYBRGreen I Master (Roche), and VEGF-A primers. The relative fold changes between stimulations were calculated with the comparative Ct method ($2^{-\delta\delta}$Ct), using β2M mRNA as the reference gene.

4.6 Cell proliferation assay

The potential mitogenic or cytotoxic effects of hormones and LPS on THP-1 monocyte cultures were evaluated with the Cell Titer 96 Cell Proliferation Assay (Promega, USA). The assay was carried out according to the manufacturer's instructions. Briefly, THP-1 monocytes (1×10^5 cells/ well) were cultured in quadruplicate in a 96-well tissue culture plate in a total volume of 100 µl culture medium either in the absence or presence of the indicated concentration of hormones or LPS for 6 h. An 8-point standard

curve of THP-1 monocytes with 2 x 10^6 cells/ml as the highest standard was produced using a 2-fold dilution series in fresh cell culture medium. Twenty µl of Owen's reagent were added to each well and cells were incubated for a further 1 h. After incubation, absorption was measured at 460 nm on a spectrophotometer (Microplate Fluorescence Reader, Bio-Rad). Cell numbers of samples were calculated by linear standard curve fitting.

4.7 Statistical analysis

Results were expressed as means ± SD from 3 independent experiments. Statistical analysis of real-time RT-PCR data was performed on ΔCt values[49] using SPSS 17.0 software. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. *P*-values were corrected for multiple comparisons with the Bonferroni-Holm method. A p-value of < 0.05 was considered significant.

5 Results

5.1 Effect of progesterone and β-estradiol on VEGF-A mRNA expression in THP-1 monocytes

To examine the hypothesis that progesterone or β-estradiol may increase the expression of growth factors in THP-1 monocytes, the mRNA expression of VEGF-A was investigated by conventional RT-PCR. As shown in Figure 1, THP-1 monocytes constitutively expressed VEGF-A mRNA. Compared to controls, a distinct up-regulation of VEGF-A mRNA expression was detected after treatment with PGE_2 and progesterone for 1 and 3 h as the intensity of VEGF-A bands were enhanced by progesterone and PGE_2 stimulation. In addition, a slight up-regulation of VEGF-A mRNA expression in β-estradiol and LPS-treated cells at 3 h stimulation was also observed. Because the conventional PCR is only semi-quantitative at best, the quantification of VEGF-A mRNA expression was then performed using real-time PCR.

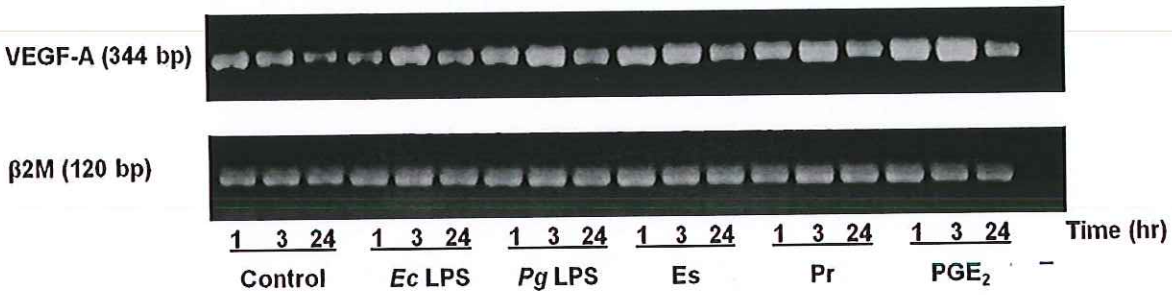


Figure 2 Effect of progesterone and β-estradiol on VEGF-A mRNA expression in THP-1 monocytes. THP-1 monocytes (4×10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), LPS from *E.coli* and *P.gingivalis* (100 ng/ml) or PGE_2 (2.5 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of VEGF-A and β2M were analyzed by RT-PCR. Positive control was PGE_2. Negative (-) control was H_2O. Shown are representative results of 3 independent experiments.

5.2 Effect of progesterone and β-estradiol on bFGF mRNA expression in THP-1 monocytes

To examine the hypothesis that progesterone or β-estradiol may modulate the expression of bFGF in THP-1 monocytes, the bFGF mRNA expression was investigated by conventional RT-PCR. As shown in **Figure 3,** THP-1 monocytes constitutively expressed bFGF mRNA. In contrast to RT-PCR products for VEGF-A, there was no clear change of bFGF mRNA expression in THP-1 monocyte treated with hormones or LPS as compared with controls. To verify these findings, the quantification of bFGF mRNA expression was then performed using real-time PCR.

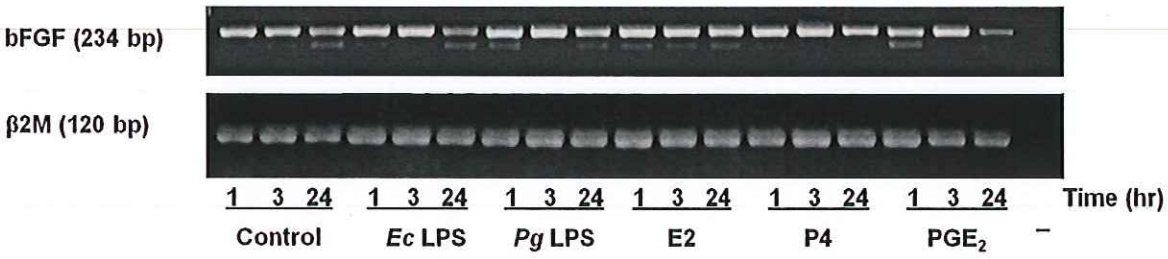


Figure 3 Effect of progesterone and β-estradiol on bFGF mRNA expression in THP-1 monocytes. THP-1 monocytes (4 x 10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), LPS from *E.coli* and *P.gingivalis* (100 ng/ml) or PGE_2 (2.5 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of bFGF and β2M were analyzed by RT-PCR. Positive control was PGE_2. Negative (-) control was H_2O. Shown are representative results of 3 independent experiments.

5.3 Effect of progesterone and β-estradiol on TGF-β mRNA expression in THP-1 monocytes

To examine the hypothesis that progesterone or β-estradiol may increase the expression of TGF-β in THP-1 monocytes, the mRNA expression of TGF-β was investigated by conventional RT-PCR. As shown in **Figure 4**, THP-1 monocytes seem not to constitutively express TGF-β mRNA. Unfortunately, there were no detectable bands of TGF-β in either of LPS or hormone stimulation. The reason for these discrepancies is unclear but a possible explanation is that LPS and female sex hormones used in the experiment may be not an inducer for TGF-β regulation in THP-1 monocytes. In addition, it should be noted that discrepancies may be also due to differences in cell types, number of cells and incubation time.

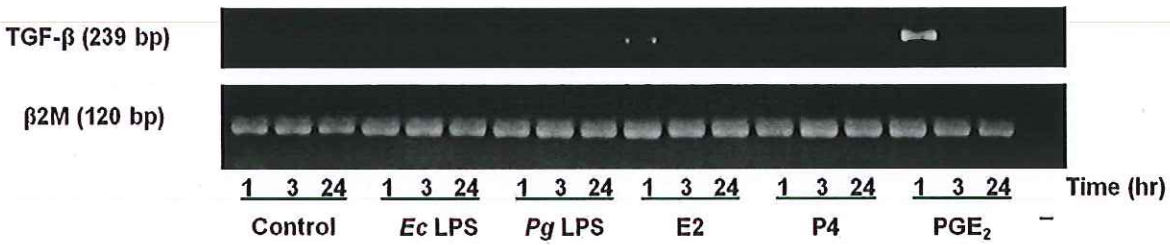


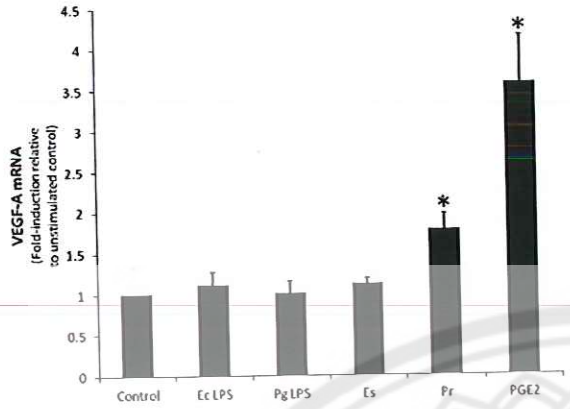
Figure 4 Effect of progesterone and β-estradiol on TGF-β mRNA expression in THP-1 monocytes. THP-1 monocytes (4×10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), LPS from *E.coli* and *P.gingivalis* (100 ng/ml) or PGE_2 (2.5 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of TGF-β and β2M were analyzed by RT-PCR. Positive control was PGE_2. Negative (-) control was H_2O. Shown are representative results of 3 independent experiments.

5.4 Real-time PCR for VEGF-A and bFGF mRNA in THP-1 monocytes

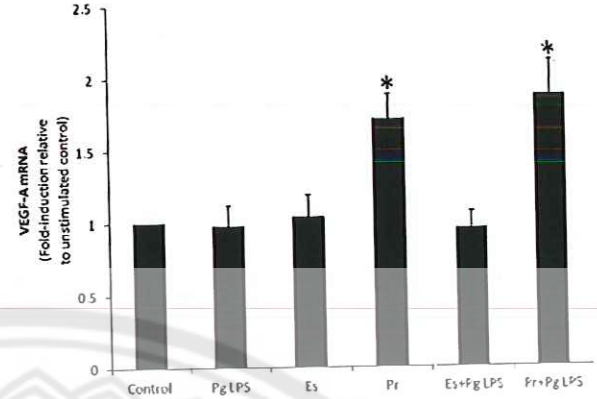
To validate the findings from conventional RT-PCR, the quantitative effect of progesterone, β-estradiol and *P.gingivalis* LPS on VEGF-A and bFGF mRNA expression was determined by real-time PCR. **Figure 5A, 5B** and 5C show the results from real-time PCR. Compared to control, both progesterone and PGE_2 significantly up-regulated VEGF-A mRNA expression (1.77- fold; $p < 0.05$ and 3.56-fold; $p < 0.001$ respectively) whereas treatment with β-estradiol or LPS from *E.coli* and *P.gingivalis* alone did not alter the VEGF-A mRNA level in THP-1 monocytes at 3 h stimulation. The expression of VEGF-A mRNA was close to 1 in β-estradiol and LPS-treated cells indicating that there were no differences in VEGF-A mRNA expression in those cells compared to control. The results from real-time PCR also confirmed the conventional RT-PCR data that bFGF mRNA is constitutively expressed in THP-1 monocytes and remains unchanged after stimulation either with female sex hormones or with bacterial LPS (**Figure 5B**). To investigate the role of β-estradiol and progesterone on VEGF-A expression by THP-1 monocytes in response to *P. gingivalis* LPS, THP-1 monocytes (0.5×10^6) were incubated with estrogen (1 ng/ml) or progesterone (10 ng/ml) in the presence and absence of LPS from *P. gingivalis* (100 ng/ml) for 3 h. Unstimulated cells and cells stimulated with hormones or LPS alone served as controls. As shown in **Figure 5C**, *P. gingivalis* LPS and β-estradiol alone had no effect on VEGF-A mRNA expression in THP-1 monocytes. Only progesterone stimulation increased VEGF-A mRNA expression by 1.71- fold compared to control ($p < 0.05$). Interestingly, progesterone, but not β-estradiol, significantly promoted VEGF-A mRNA expression in *P. gingivalis* LPS treated cells (*P. gingivalis* LPS; 0.97-fold vs progesterone plus *P. gingivalis* LPS 1.87-fold, $p < 0.05$). Whether up-regulation of VEGF-A mRNA expression was due to mitogenic or cytotoxic effect of progesterone on the viability of THP-1 monocytes was also determined. After 6 h of β-estradiol or progesterone stimulation, no significant change in the cell number of THP-1 monocytes was

detected in comparison to the control ($p > 0.05$, **Figure 6**). In contrast, cell numbers of THP-1 monocytes stimulated with *E. coli* or *P. gingivalis* LPS were significantly lower ($p < 0.05$) compared to control.

A)



B)



C)

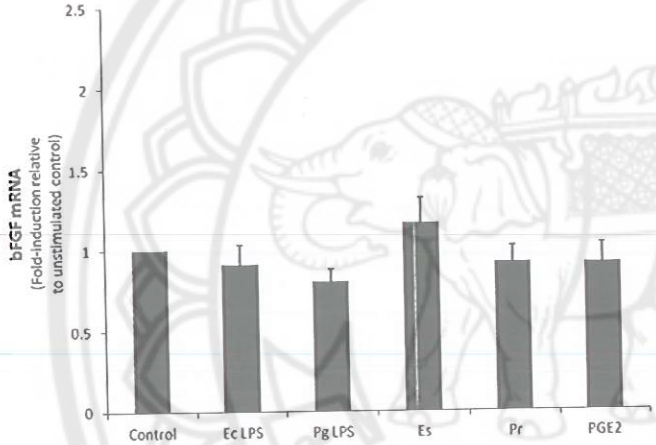


Figure 5 VEGF-A and bFGF mRNA expression in THP-1 monocytes after stimulation with β-estradiol, progesterone and *P.gingivalis* LPS. A) and B) THP-1 monocytes (4×10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), PGE_2 (2.5 ng/ml) and/or LPS from *E.coli* and *P.gingivalis* (100 ng/ml) for 3h. C) THP-1 monocytes were stimulated with a combination of progesterone or β-estradiol and *P.gingivalis* LPS for 3 h, hormones or LPS alone served as controls. The amount of VEGF-A and bFGF mRNA was quantified by real-time PCR. The data are expressed as mean of 3 independent experiments. *: $p < 0.05$ compared with controls.

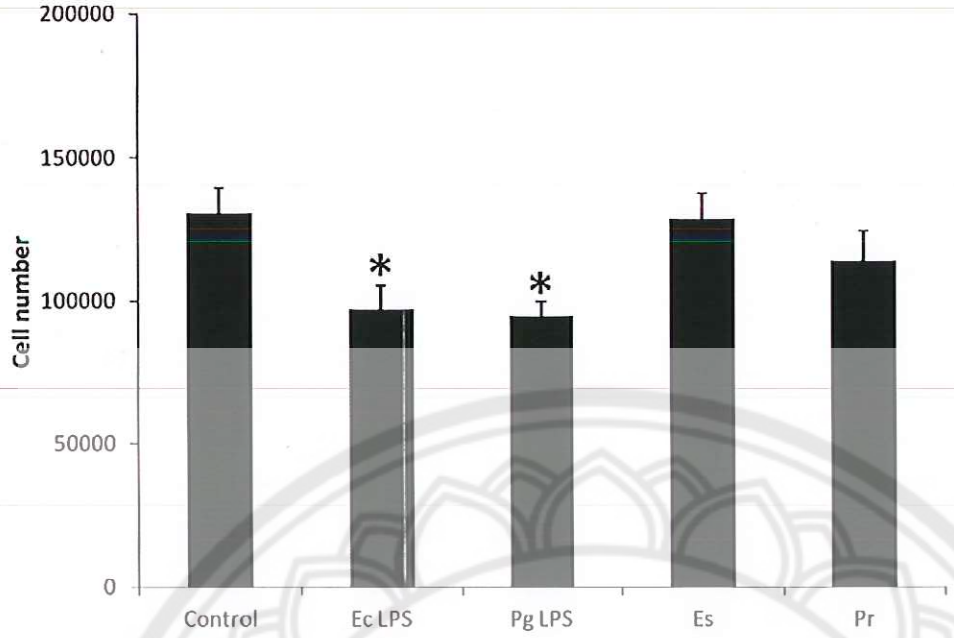


Figure 6 The effect of β-estradiol and progesterone on cell proliferation in THP-1 monocytes. THP-1 monocytes (1 x 10^5 cells/well) were stimulated with β-estradiol; Es (1 ng/ml), progesterone; Pr (10 ng/ml) or LPS from *E.coli* and *P.gingivalis* (100 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 6 h. The graph represents mean ± SD (n = 4). *: $p < 0.05$ compared with controls.

6 Discussion

Since female sex hormones play a role in the pathogenesis of pregnancy-associated gingival diseases [8, 50], understanding the effect of estrogen and progesterone on cellular immune responses may provide an insight in the mechanistic link between pregnancy and periodontal diseases. Although studies have demonstrated the pro-inflammatory roles of female sex hormones in pregnancy gingivitis and pregnancy tumor [17, 50, 51], the underlying molecular mechanisms in this pathway are not fully understood. The present study demonstrated that progesterone, but not estradiol, increased VEGF-A mRNA expression in THP-1 monocytes. In addition, progesterone significantly enhanced VEGF-A mRNA expression in *P. gingivalis* LPS treated monocytes in comparison with a treatment with *P. gingivalis* LPS alone. However, we observed that either estradiol or progesterone had no or little effect on bFGF production at mRNA levels. These data may reflect that although monocytes are suggested to be one of a major source for bFGF, the regulation of this gene at transcriptional levels may be not associated with the stimulation by bacterial LPS and hormones in THP-1 monocytes. It is therefore interesting to explore the transcriptional regulation of bFGF in other possible cell types such as gingival fibroblasts and macrophages[52].

The results that progesterone enhances VEGF-A mRNA expression in THP-1 monocytes support the concept that female sex hormones modify the periodontal inflammation and healing process during pregnancy. VEGF, a member of angiogenic factor family, plays a vital role in the process of vascular formation [53]. It has been suggested that elevated levels of VEGF are associated with the pathological angiogenesis such as tumors, rheumatoid arthritis and diabetic retinopathy [53, 54]. Macrophages/monocytes are one of a major source of VEGF [55]. The known inducers for VEGF are platelet-derived growth factor, phorbol esters, IL-1β and PGE_2 [56, 57]. Similar to a previous study[56], we found that PGE_2 (which was a positive control) up-regulated VEGF-A mRNA expression in THP-1 monocytes. Estradiol and progesterone have been reported to regulate the production of several growth factors such as VEGF-A and nerve growth factor in monocytes/macrophages [20, 52, 58]. At protein levels, estradiol and progesterone can stimulate VEGF-A secretion by U937 monocytes [52] and peritoneal macrophages [59]. In addition, Kanda and

Watanabe (2002) found that 17β-estradiol induced VEGF-A production at both mRNA and protein levels in phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 monocytes [58]. However, the present study demonstrated that only progesterone significantly up-regulated VEGF-A mRNA expression whereas estradiol had no or very little effect on the VEGF-A mRNA expression. These discrepancies may be explained at least in part by the diverse immunological assay variations, different concentrations of hormones or different cell differentiation techniques[60]. Indeed, PMA-treated THP-1 monocytes in the previous study were differentiated into macrophage-like cells[58] while the present study used VitD3 to differentiate THP-1 monocytes into mature monocyte characteristics.

In response to LPS stimulation, female sex hormones display an enhancing effect on cytokine or growth factor production in various cell types. In human gingival fibroblasts, Yokoyama et al. (2005) reported that estradiol increases sensitivity to *Campylobacter rectus*-induced VEGF-A secretion [61]. In addition, progesterone has been shown to enhance the production of IL-1β and IL-8, but to inhibit TNF-α production by monocytes stimulated with *E.coli* LPS [62]. Moreover, progesterone promotes *E.coli* LPS-induced VEGF-A secretion in U937 monocytes [52]. Interestingly, the present study demonstrated that treatment with progesterone led to a significant increase in the *P. gingivalis* LPS-stimulated expression of VEGF-A mRNA by THP-1 monocytes. Taken together, these data suggest the pro-inflammatory role of female sex hormones in cellular immune response to bacterial LPS. In addition, the interaction between progesterone and *P. gingivalis* LPS may have a regulatory role in the early pathological vascular changes in pregnancy gingivitis.

The results from cell proliferation assay showed that both estradiol and progesterone had no proliferative or cytotoxic effects on THP-1 monocytes. Any progesterone-induced changes in VEGF-A expression were therefore not due to changes in cell numbers. However, it should be noted that cell numbers of THP-1 monocyte stimulated with LPS were significantly lower in comparison to control indicating that LPS may have a cytotoxic effect on the THP-1 monocytes at 6 h. However, LPS-stimulation of THP-1 monocytes or stimulation with other pro-inflammatory agents such as PMA induced cell differentiation and growth cycle

arrest [63]. Therefore, it is possible that the unstimulated THP-1 monocytes continued to proliferate while LPS-stimulated cells switched from proliferation to differentiation. In addition, although cell numbers were lower after LPS-stimulation in THP-1 monocytes in comparison to control, the remaining cell number was still comparable to the starting cell number of the experimental setup. This likely indicates a decline in the proliferative capacity rather than LPS-induced cell death. In conclusion, the present study supports that progesterone modulates cellular immune responses through growth factor up-regulation. The effect of progesterone on VEGF-A synthesis may play a role in periodontal disease asscociated with pregnancy.

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   ชื่อเรื่องที่ตีพิมพ์ : Progesterone, but not β-estradiol, enhances *P.gingivalis* LPS induced-VEGF-A expression in human THP-1 monocytes.

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Progesterone, but not β-estradiol, enhances *P.gingivalis* LPS induced-VEGF-A expression in human THP-1 monocytes.

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Running title: Progesterone enhances the expression of VEGF-A

Abstract

Background/purpose: Progesterone and estrogen are elevated during pregnancy and play a role in maternal immune responses. In addition, unbalanced metabolism of growth factors has been demonstrated in pregnancy tumor. Therefore, we aimed to investigate the effect of progesterone and β-estradiol on vascular endothelial growth factor-A (VEGF-A) and basic fibroblast growth factor (bFGF) mRNA expression in THP-1 monocytes in response to *Porphyromonas gingivalis* lipopolysaccharide (LPS).

Materials and methods: THP-1 monocytes were incubated with progesterone, β-estradiol or LPS from *Escherichia coli* and *P.gingivalis* for up to 24 h. The expression for VEGF-A and bFGF was investigated using conventional RT-PCR and real-time RT-PCR. Cell growth was assessed using cell proliferation assay.

Results: We reported herein that progesterone, but not β-estradiol, increased VEGF-A mRNA expression in THP-1 monocytes. Significantly, progesterone enhanced VEGF-A mRNA expression in *P. gingivalis* LPS treated monocytes in comparison with a treatment with *P. gingivalis* LPS alone. However, neither β-estradiol nor progesterone had any effect on bFGF production at mRNA levels.

Conclusion: The enhancing effect of progesterone on VEGF-A mRNA expression may have a role in the pathogenesis of pyogenic granuloma in pregnant women.

Keywords: Progesterone; estrogen; VEGF-A; *P.gingivalis*; monocytes

Introduction

Pregnancy tumor is a term for pyogenic granuloma that occurs on the gingival mucosa of pregnant women in response primarily to local irritation or injury [1-3]. Although the clinical and histological characteristics of pregnancy tumor are well documented [3], its etiology and pathogenesis are not fully understood. Hormonal imbalance is thought to be a major determinant that is responsible for the development of gingival hyperreactive inflammatory responses [1, 4]. Indeed, it has been postulated that the pathogenesis of pregnancy gingivitis is initiated by bacterial biofilm and exacerbated by endogenous sex steroid hormones [5]. The levels of serum and salivary sex hormones such as progesterone and estrogen are significantly increased throughout the 3 trimesters of pregnancy[6, 7]. Clinical studies have shown the biological link between increased gingival inflammation during pregnancy and changes in the local immune system[6, 8-10]. Recently, Gursoy et al. (2012) have demonstrated a positive association between increased level of salivary estrogen and the severity of gingival inflammation during pregnancy [4]. Progesterone and estrogen have been shown to stimulate the production of prostaglandin E_2 (PGE_2) [11] but to inhibit the production of interleukin 1β (IL-1β) from human peripheral monocytes [12]. Another study showed that estrogen reduced the expression of chemokine CCL3 mRNA but increased the expression of CCL5 by periodontal ligament cells [13]. These data suggest that alterations in hormone levels and host inflammatory response as a result of pregnancy may lead to further dysregulation of immune-inflammatory responses in the periodontium, causing periodontal inflammation [6, 14, 15].

Histological studies have revealed that pyogenic granuloma is characterized by the up-regulation of growth factors such as vascular endothelial growth factor (VEGF) and basic

fibroblast growth factor (bFGF) suggesting that the unbalanced/excessive production of growth factors may be one mechanism that mediates the development of pyogenic granuloma [1, 16]. *In vitro*, it has been shown that female sex hormone is a potential regulator of the production of several growth factors such as VEGF, bFGF and nerve growth factor in different cell types [17-20]. It is thus plausible that female sex hormone may modulate the response to periodontal infection in term of growth factor synthesis in pregnancy-related pyogenic granuloma. Since *P. gingivalis* is a major bacterial species implicated in many forms of gingival diseases, including pregnancy gingivitis[21, 22], the interaction between female sex hormones and *P. gingivalis* may be fundamentally important in the development of pregnancy related-pyogenic granuloma. In addition, only few studies have demonstrated the interacting role between sex hormones and oral bacteria such as *P. gingivalis* in the synthesis of growth factors by different cells in gingival tissues [23, 24]. Therefore, the aim of this study was to investigate the *in vitro* effect of estrogen and progesterone on the expression of VEGF-A and bFGF at mRNA level in differentiated THP-1 monocyte treated with *P. gingivalis* LPS.

Materials and Methods

Materials

Progesterone (P7556), estrogen (β-Estradiol, E4389) and cell culture media were purchased from Sigma-Aldrich (St. Louis, USA). All plasticware were purchased from (Nunc A/S, Denmark). Vitamin D_3 (1α, 25-dihydroxy-vitamin D_3) was purchased from Calbiochem (Merck Chemicals, USA). Ultrapure LPS from *E. coli* 0111.B4 and ultrapure LPS from *P. gingivalis* were purchased from Invivogen (San Diego, USA). Recombinant prostaglandin E_2 (PGE_2) was purchased from R&D Systems (Minneapolis, USA).

THP-1 monocytes cell culture and stimulation

THP-1 pro-monocytes were purchased from the Cell Line Service (CLS, Germany) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2. Cells were maintained at a density of 3-8 x 10^5 cells/ml and cell viability was monitored using trypan blue exclusion, which was always >95%. THP-1 cells have a pro-monocyte phenotype with low levels of CD14 expression. Therefore prior to use in stimulation experiments we differentiated THP-1 promonocytes to a monocyte phenotype by incubation with 0.1 µM vitamin D_3 (VitD3) for 48 h, as previously reported[25]. It has shown that 100 ng/ml *E.coli/P.gingivalis* LPS is an optimal concentration used in stimulation experiments[25] and progesterone, β-estradiol and PGE_2 concentrations used in experiments are chosen based on previous studies applying a comparable experimental setup[11]. Cells were then stimulated with LPS from *E. coli* and *P. gingivalis* (100 ng/ml), progesterone (10 ng/ml), β-estradiol (1 ng/ml) or PGE_2 (2.5 ng/ml) for 1, 3 and 24 h.

RNA extraction and cDNA synthesis by Reverse Transcription

Total RNA was isolated from culture cells using a commercial RNA extraction kit (RNeasy® Mini Kit, Qiagen, Germany) according to the manufacturer's instruction. The total RNA concentration of each sample was measured with a spectrophotometer (Nanodrop, Thermo Fisher, USA) and then stored at -80 °C. An aliquot containing 1.0 µg of total RNA was used for the reverse transcription reaction, which was conducted using iScript™ cDNA Synthesis kit (Bio-Rad, USA). cDNA samples were then stored at 2-8 °C.

Conventional RT-PCR

The mRNA expression of VEGF-A and bFGF genes was detected using a conventional PCR method. β2 microglobulin (β2M) was used as an internal control gene. All primers used for PCR analysis were purchased from Intron Bio (Korea). The primer sets specific for each gene and the PCR conditions are summarized in **Table 1.** The PCR was performed using i-Taq™ PCR Master mix (Intron Bio, Korea) with 2.0 µl of cDNA sample. PCR amplification was conducted for 35 cycles and the products of the PCR were then separated by 2% agarose gel electrophoresis, visualized with ethidium bromide staining, and viewed under UV light.

Table 1 Primers used in the polymerase chain reaction expression analyses

Gene	*Primer sequence (5'-3')*	*Annealing Temp. (°C)*	*Product size (bp)*
VEGF-A [26]	F –ATGAACTTTCTGCTGTCTTGGGT	60	344
	R –TGGCCTTGGTGAGGTTTGATCC		
bFGF [27]	F –AGAGCGACCCTCACATCAAG	60	234
	R –ACTGCCCAGTTCGTTTCAGT		
β2M	F –ACCCCCACTGAAAAAGATGA	60	120
	R –CTTATGCACGCTTAACTATC		

Quantitative analyses for VEGF-A mRNA using real-time RT-PCR

The quantification of VEGF-A mRNA levels was performed using a LightCycler® 480II detection system (Roche). Reaction mixtures for PCR (20 µl) were prepared by mixing 1.0 µl of cDNA solution, LightCycler 480 SYBRGreen I Master (Roche), and VEGF-A primers. The relative fold changes between stimulations were calculated with the comparative Ct method ($2^{-\delta\delta}$Ct), using β2M mRNA as the reference gene.

Cell proliferation assay

The potential mitogenic or cytotoxic effects of hormones and LPS on THP-1 monocyte cultures were evaluated with the Cell Titer 96 Cell Proliferation Assay (Promega, USA). The assay was carried out according to the manufacturer's instructions. Briefly, THP-1 monocytes (1×10^5 cells/ well) were cultured in quadruplicate in a 96-well tissue culture plate in a total volume of 100 µl culture medium either in the absence or presence of the indicated concentration of hormones or LPS for 6 h. An 8-point standard curve of THP-1 monocytes with 2×10^6 cells/ml as the highest standard was produced using a 2-fold dilution series in fresh cell culture medium.

Twenty µl of Owen's reagent were added to each well and cells were incubated for a further 1 h. After incubation, absorption was measured at 460 nm on a spectrophotometer (Microplate Fluorescence Reader, Bio-Rad). Cell numbers of samples were calculated by linear standard curve fitting.

Statistical analysis

Results were expressed as means ± SD from 3 independent experiments. Statistical analysis of real-time RT-PCR data was performed on ΔCt values[28] using SPSS 17.0 software. Shapiro-Wilk testing for normal distribution and Levene testing for homogeneity of variance were performed prior to ANOVA. *P*-values were corrected for multiple comparisons with the Bonferroni-Holm method. A p-value of < 0.05 was considered significant.

Results

Effect of progesterone and β-estradiol on VEGF-A mRNA expression in THP-1 monocytes

To examine the hypothesis that progesterone or β-estradiol may increase the expression of growth factors in THP-1 monocytes, the mRNA expression of two growth factors were investigated by conventional RT-PCR. As shown in **Figure 1**, THP-1 monocytes constitutively expressed VEGF-A and bFGF mRNA. Compared to controls, a distinct up-regulation of VEGF-A mRNA expression was detected after treatment with PGE_2 and progesterone for 1 and 3 h as the intensity of VEGF-A bands were enhanced by progesterone and PGE_2 stimulation. In addition, a slight up-regulation of VEGF-A mRNA expression in β-estradiol and LPS-treated cells at 3 h stimulation was also observed. In contrast, there was no clear change of bFGF mRNA expression in THP-1 monocyte treated with hormones or LPS as compared with controls. Because the conventional PCR is only semi-quantitative at best, the quantification of VEGF-A mRNA expression was then performed using real-time PCR.

Real-time PCR for VEGF-A mRNA in THP-1 monocytes

To validate the findings from conventional RT-PCR, the quantitative effect of progesterone, β-estradiol and *P.gingivalis* LPS on VEGF-A and bFGF mRNA expression was determined by real-time PCR. **Figure 2A**, **2B** and **2C** show the results from real-time PCR. Compared to control, both progesterone and PGE_2 significantly up-regulated VEGF-A mRNA expression (1.77- fold; $p < 0.05$ and 3.56-fold; $p < 0.001$ respectively) whereas treatment with β-estradiol or LPS from *E.coli* and *P.gingivalis* alone did not alter the VEGF-A mRNA level in THP-1 monocytes at 3 h stimulation. The expression of VEGF-A mRNA was close to 1 in β-estradiol and LPS-treated cells indicating

that there were no differences in VEGF-A mRNA expression in those cells compared to control. The results from real-time PCR also confirmed the conventional RT-PCR data that bFGF mRNA is constitutively expressed in THP-1 monocytes and remains unchanged after stimulation either with female sex hormones or with bacterial LPS (**Figure 2B**). To investigate the role of β-estradiol and progesterone on VEGF-A expression by THP-1 monocytes in response to *P. gingivalis* LPS, THP-1 monocytes (0.5×10^6) were incubated with estrogen (1 ng/ml) or progesterone (10 ng/ml) in the presence and absence of LPS from *P. gingivalis* (100 ng/ml) for 3 h. Unstimulated cells and cells stimulated with hormones or LPS alone served as controls. As shown in **Figure 2C**, *P. gingivalis* LPS and β-estradiol alone had no effect on VEGF-A mRNA expression in THP-1 monocytes. Only progesterone stimulation increased VEGF-A mRNA expression by 1.71- fold compared to control ($p < 0.05$). Interestingly, progesterone, but not β-estradiol, significantly promoted VEGF-A mRNA expression in *P. gingivalis* LPS treated cells (*P. gingivalis* LPS; 0.97-fold vs progesterone plus *P. gingivalis* LPS 1.87-fold, $p < 0.05$). Whether up-regulation of VEGF-A mRNA expression was due to mitogenic or cytotoxic effect of progesterone on the viability of THP-1 monocytes was also determined. After 6 h of β-estradiol or progesterone stimulation, no significant change in the cell number of THP-1 monocytes was detected in comparison to the control ($p > 0.05$, **Figure 3**). In contrast, cell numbers of THP-1 monocytes stimulated with *E. coli* or *P. gingivalis* LPS were significantly lower ($p < 0.05$) compared to control.

Discussion

Since female sex hormones play a role in the pathogenesis of pregnancy-associated gingival diseases [2, 29], understanding the effect of estrogen and progesterone on cellular immune responses may provide an insight in the mechanistic link between pregnancy and periodontal diseases. Although studies have demonstrated the pro-inflammatory roles of female sex hormones in pregnancy gingivitis and pregnancy tumor [4, 29, 30], the underlying molecular mechanisms in this pathway are not fully understood. The present study demonstrated that progesterone, but not estradiol, increased VEGF-A mRNA expression in THP-1 monocytes. In addition, progesterone significantly enhanced VEGF-A mRNA expression in *P. gingivalis* LPS treated monocytes in comparison with a treatment with *P. gingivalis* LPS alone. However, we observed that either estradiol or progesterone had no or little effect on bFGF production at mRNA levels. These data may reflect that although monocytes are suggested to be one of a major source for bFGF, the regulation of this gene at transcriptional levels may be not associated with the stimulation by bacterial LPS and hormones in THP-1 monocytes. It is therefore interesting to explore the transcriptional regulation of bFGF in other possible cell types such as gingival fibroblasts and macrophages[15].

The results that progesterone enhances VEGF-A mRNA expression in THP-1 monocytes support the concept that female sex hormones modify the periodontal inflammation and healing process during pregnancy. VEGF, a member of angiogenic factor family, plays a vital role in the process of vascular formation [31]. It has been suggested that elevated levels of VEGF are associated with the pathological angiogenesis such as tumors, rheumatoid arthritis and diabetic retinopathy [31, 32]. Macrophages/monocytes are one of a major source of VEGF [33]. The known

inducers for VEGF are platelet-derived growth factor, phorbol esters, IL-1β and PGE_2 [34, 35]. Similar to a previous study[34], we found that PGE_2 (which was a positive control) up-regulated VEGF-A mRNA expression in THP-1 monocytes. Estradiol and progesterone have been reported to regulate the production of several growth factors such as VEGF-A and nerve growth factor in monocytes/macrophages [15, 19, 36]. At protein levels, estradiol and progesterone can stimulate VEGF-A secretion by U937 monocytes [15] and peritoneal macrophages [37]. In addition, Kanda and Watanabe (2002) found that 17β-estradiol induced VEGF-A production at both mRNA and protein levels in phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 monocytes [36]. However, the present study demonstrated that only progesterone significantly up-regulated VEGF-A mRNA expression whereas estradiol had no or very little effect on the VEGF-A mRNA expression. These discrepancies may be explained at least in part by the diverse immunological assay variations, different concentrations of hormones or different cell differentiation techniques[38]. Indeed, PMA-treated THP-1 monocytes in the previous study were differentiated into macrophage-like cells[36] while the present study used VitD3 to differentiate THP-1 monocytes into mature monocyte characteristics.

In response to LPS stimulation, female sex hormones display an enhancing effect on cytokine or growth factor production in various cell types. In human gingival fibroblasts, Yokoyama et al. (2005) reported that estradiol increases sensitivity to *Campylobacter rectus*-induced VEGF-A secretion [24]. In addition, progesterone has been shown to enhance the production of IL-1β and IL-8, but to inhibit TNF-α production by monocytes stimulated with *E.coli* LPS [39]. Moreover, progesterone promotes *E.coli* LPS-induced VEGF-A secretion in U937 monocytes [15]. Interestingly, the present study demonstrated that treatment with progesterone led to a

significant increase in the *P. gingivalis* LPS-stimulated expression of VEGF-A mRNA by THP-1 monocytes. Taken together, these data suggest the pro-inflammatory role of female sex hormones in cellular immune response to bacterial LPS. In addition, the interaction between progesterone and *P. gingivalis* LPS may have a regulatory role in the early pathological vascular changes in pregnancy gingivitis.

The results from cell proliferation assay showed that both estradiol and progesterone had no proliferative or cytotoxic effects on THP-1 monocytes. Any progesterone-induced changes in VEGF-A expression were therefore not due to changes in cell numbers. However, it should be noted that cell numbers of THP-1 monocyte stimulated with LPS were significantly lower in comparison to control indicating that LPS may have a cytotoxic effect on the THP-1 monocytes at 6 h. However, LPS-stimulation of THP-1 monocytes or stimulation with other pro-inflammatory agents such as PMA induced cell differentiation and growth cycle arrest [40]. Therefore, it is possible that the unstimulated THP-1 monocytes continued to proliferate while LPS-stimulated cells switched from proliferation to differentiation. In addition, although cell numbers were lower after LPS-stimulation in THP-1 monocytes in comparison to control, the remaining cell number was still comparable to the starting cell number of the experimental setup. This likely indicates a decline in the proliferative capacity rather than LPS-induced cell death. In conclusion, the present study supports that progesterone modulates cellular immune responses through growth factor up-regulation. The effect of progesterone on VEGF-A synthesis may play a role in periodontal disease asscociated with pregnancy.

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Figure legends

Figure 1 Effect of progesterone and β-estradiol on VEGF-A and bFGF mRNA expression in THP-1 monocytes. THP-1 monocytes (4 x 10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), LPS from *E.coli* and *P.gingivalis* (100 ng/ml) or PGE_2 (2.5 ng/ml) for 1-24 h. Total RNA was isolated and the mRNA expression of VEGF-A, bFGF and β2M were analyzed by RT-PCR. Positive control was PGE_2. Negative (-) control was H_2O. Shown are representative results of 3 independent experiments.

Figure 2 VEGF-A and bFGF mRNA expression in THP-1 monocytes after stimulation with β-estradiol, progesterone and *P.gingivalis* LPS. A) and B) THP-1 monocytes (4 x 10^6 cells) were cultured in the absence or presence of progesterone; Pr (10 ng/ml), β-estradiol; Es (1 ng/ml), PGE_2 (2.5 ng/ml) and/or LPS from *E.coli* and *P.gingivalis* (100 ng/ml) for 3h. C) THP-1 monocytes were stimulated with a combination of progesterone or β-estradiol and *P.gingivalis* LPS for 3 h, hormones or LPS alone served as controls. The amount of VEGF-A and bFGF mRNA was quantified by real-time PCR. The data are expressed as mean of 3 independent experiments. *: $p < 0.05$ compared with controls.

Figure 3 The effect of β-estradiol and progesterone on cell proliferation in THP-1 monocytes. THP-1 monocytes (1 x 10^5 cells/well) were stimulated with β-estradiol; Es (1 ng/ml), progesterone; Pr (10 ng/ml) or LPS from *E.coli* and *P.gingivalis* (100 ng/ml). Cell proliferation was analysed with the Cell Titer 96 cell proliferation assay after 6 h. The graph represents mean ± SD (n = 4). *: $p < 0.05$ compared with controls.

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Figure 1

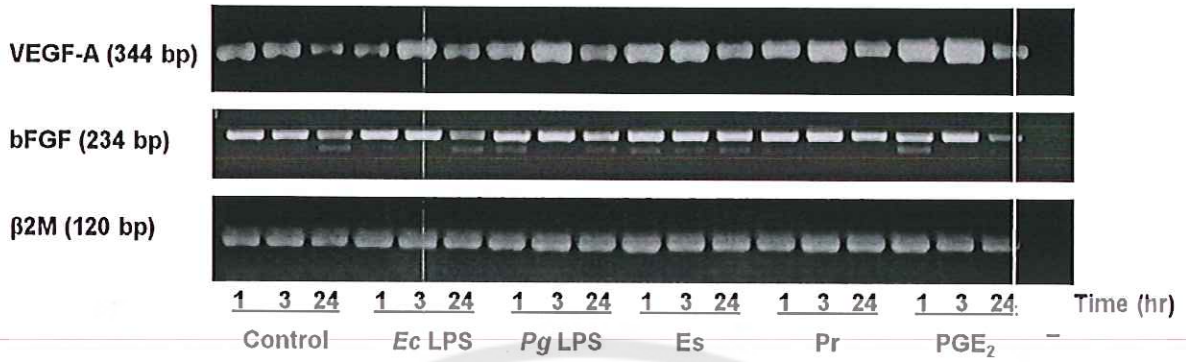

VEGF-A (344 bp)
bFGF (234 bp)
β2M (120 bp)
1 3 24 1 3 24 1 3 24 1 3 24 1 3 24 1 3 24
Time (hr)
Control
Ec LPS
Pg LPS
Es
Pr
PGE2

Figure2 A)

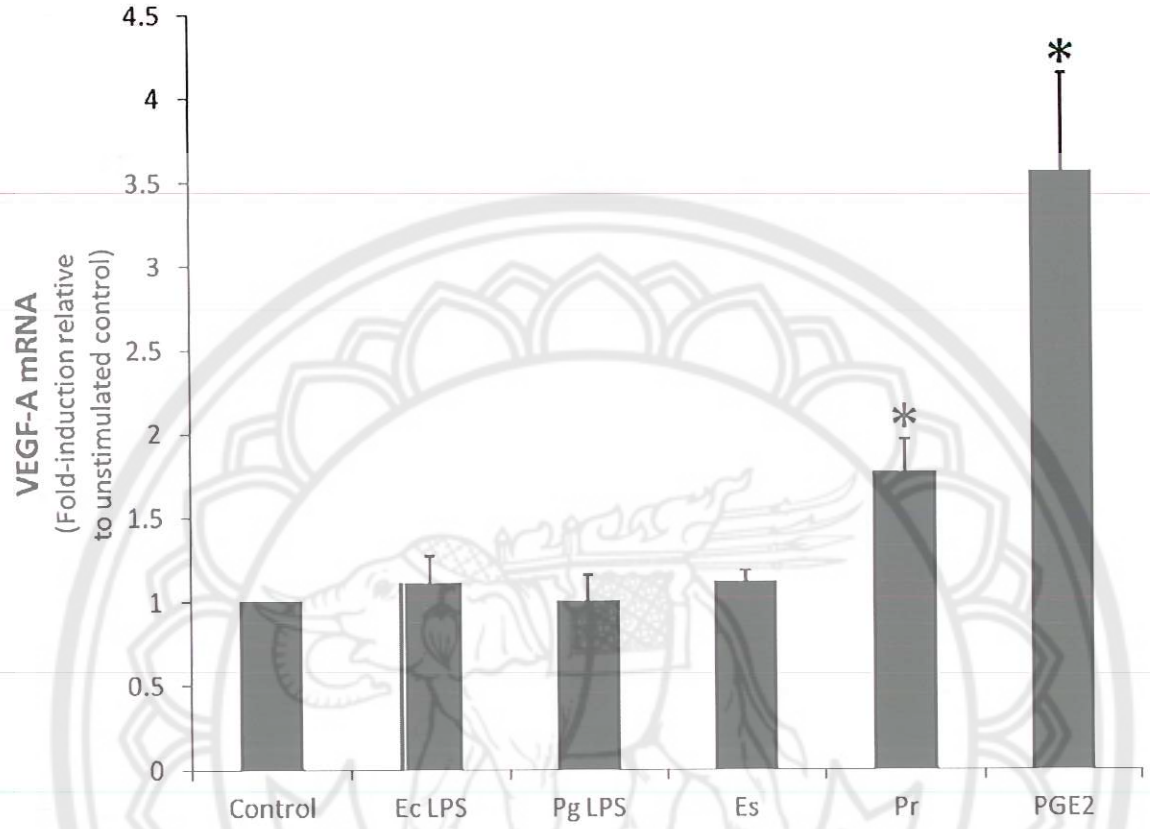
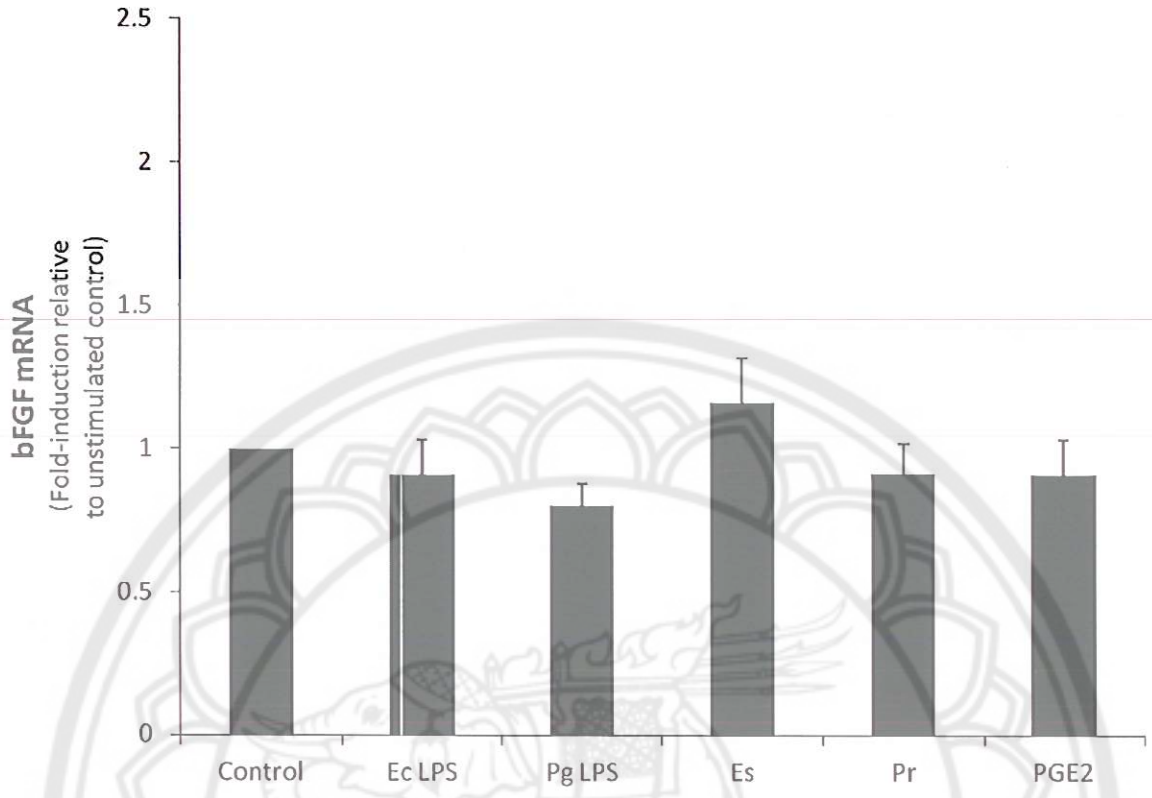

VEGF-A mRNA
(Fold-induction relative to unstimulated control)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Control
Ec LPS
Pg LPS
Es
Pr
PGE2

Figure2 B)


bFGF mRNA
(Fold-induction relative to unstimulated control)
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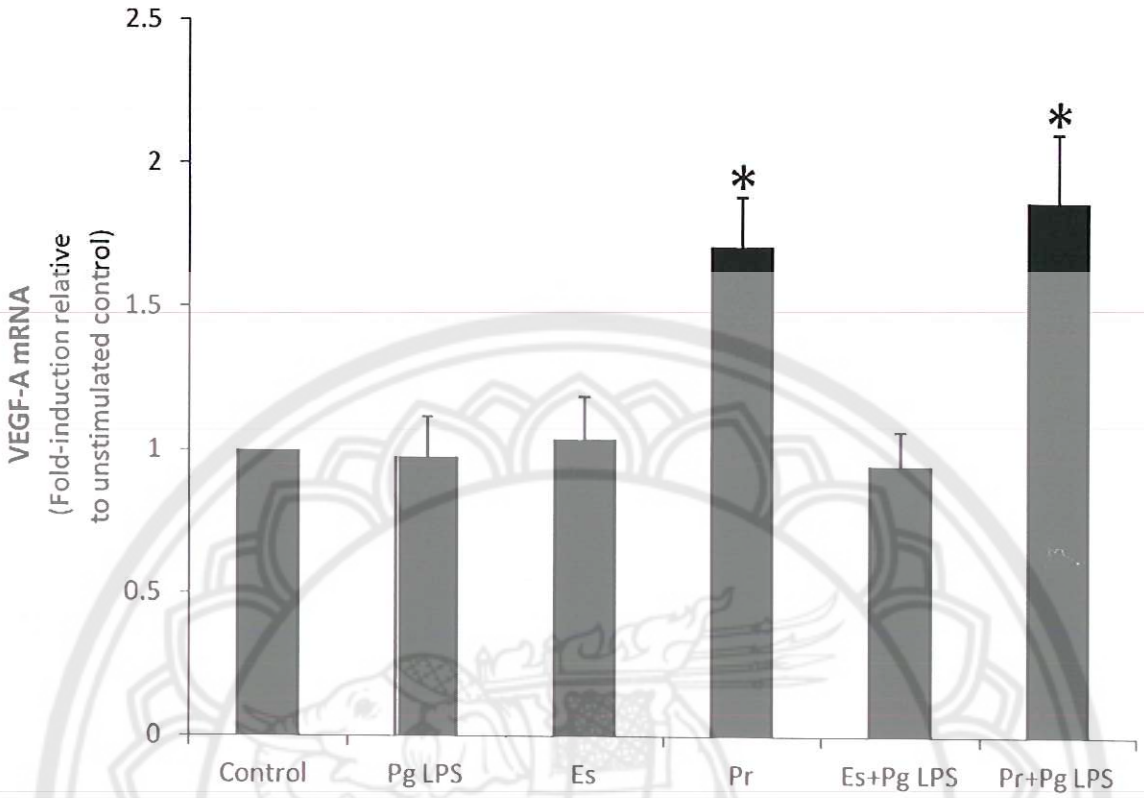
2
1.5
1
0.5
0
Control
Ec LPS
Pg LPS
Es
Pr
PGE2

Figure 2C


VEGF-A mRNA
(Fold-induction relative to unstimulated control)
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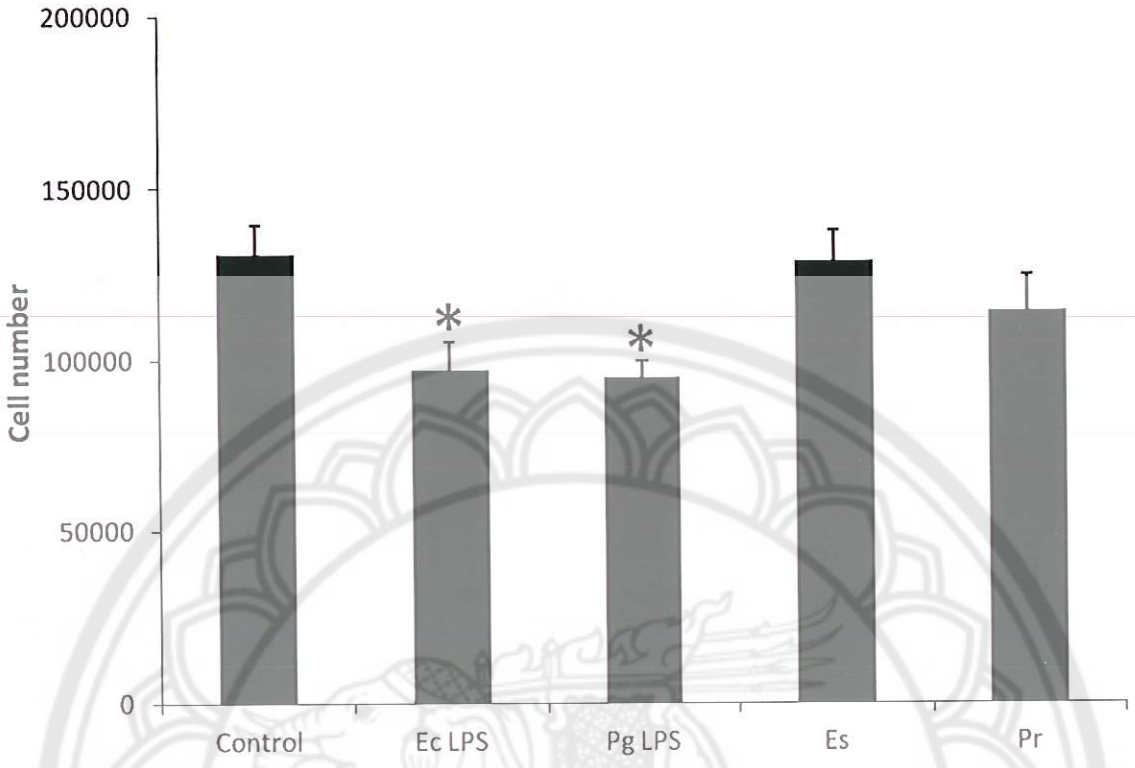
2
1.5
1
0.5
0
*
*
Control
Pg LPS
Es
Pr
Es+Pg LPS
Pr+Pg LPS

Figure 3


200000
150000
100000
50000
0
Cell number
*
*
Control
Ec LPS
Pg LPS
Es
Pr

เลขทะเบียน.....

หนังสือยินยอมการเผยแพร่ผลงานทางวิชาการบนเว็บไซต์ ฐานข้อมูล NU Digital Repository (http://obj.lib.nu.ac.th/media/) สำนักหอสมุด มหาวิทยาลัยนเรศวร

ตามที่ข้าพเจ้า ทพ.ดร.ไพบูลย์ จิตประเสริฐวงศ์ (ภาควิชาทันตกรรมป้องกัน คณะทันตแพทยศาสตร์) ได้ส่งผลงานทางวิชาการการรายงานการวิจัย (เรื่อง) รายงานวิจัยฉบับสมบูรณ์ผลของฮอร์โมนเพศหญิงเอสโตรเจนต่อการแสดงออกของยีนสารกระตุ้นการเติบโตในเซลล์เม็ดเลือดขาวชนิดโมโนไซต์ ทีเอชพี-วัน

ปีที่พิมพ์ 2556

ข้าพเจ้าขอรับรองว่า ผลงานทางวิชาการเป็นลิขสิทธิ์ของข้าพเจ้า ทพ.ดร.ไพบูลย์ จิตประเสริฐวงศ์ (ผู้วิจัยร่วม) และท่านอื่น ๆ เป็นเจ้าของลิขสิทธิ์ร่วม และเพื่อให้ผลงานทางวิชาการของข้าพเจ้าเป็นประโยชน์ต่อการศึกษาและสาธารณชน จึงอนุญาตให้เผยแพร่ผลงาน ดังนี้

- [x] อนุญาตให้เผยแพร่
- [ ] ไม่อนุญาตให้เผยแพร่ เนื่องจาก.....

.....

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ลงชื่อ .....

(ผศทพ.ดร.ไพบูลย์ จิตประเสริฐวงศ์)

วันที่ 3 พ.ย 58

หมายเหตุ ลิขสิทธิ์ใดๆ ที่ปรากฏอยู่ในผลงานนี้เป็นความรับผิดชอบของเจ้าของผลงาน ไม่ใช่ของสำนักหอสมุด